BIOCHEMISTRY OF SUBMARINE AND DIVING STRESS
III. PLASMA CREATINE, CREATINE PHOSPHATE AND CREATINE PHOSPHOKINASE RESPONSES TO HYPERCAPNIA

by

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SUMMARY PAGE

PROBLEM

To evaluate the usefulness of the measurement of plasma creatine phosphokinase activity as an indicator of the stress of high environmental carbon dioxide in which Navy divers, submariners or other personnel may be required to work.

FINDINGS

Leakage of creatine phosphokinase into the plasma has been shown to occur in experimental animals in response to carbon dioxide stress. The increase in plasma enzyme activity corresponds closely to the degree of stress experienced by the animals.

APPLICATION

It is possible to employ an analysis of plasma creatine phosphokinase activity to evaluate the severity of carbon dioxide stress in experimental animals. Since the test provides a clear-cut signal indicating potentially serious metabolic derangement by an environmental stress, it may logically be applied as a stress indicator under operational situations in which Navy divers or other persons work in closed or restricted environments.

ADMINISTRATIVE INFORMATION

This investigation was conducted as part of Bureau of Medicine and Surgery Work Unit MF12.524.006-9028BA9K - Time Concentration Exposure Limits of CO_2 in Navy Submarine and Diving Facilities. The present report is number 10 on this Work Unit. The manuscript was approved for publication on 14 April 1971 and designated as Naval Submarine Medical Research Laboratory Report Number 661.

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ABSTRACT

Creatine phosphokinase, the enzyme which is currently the most sensitive tool for detecting myocardial infarction, has been shown to also be useful for the detection of serious or irreversible damage to animals exposed to stressful levels of environmental carbon dioxide. Since accumulation of carbon dioxide is a problem for which Navy divers must be constantly alert and may affect workers in any confined environment, this analysis is proposed as a clinical test for monitoring potential damage to the health of operational personnel.

BIOCHEMISTRY OF SUBMARINE AND DIVING STRESS:

III. Plasma Creatine, Creatine Phosphate and Creatine Phosphokinase Responses to Hypercapnia

INTRODUCTION

Adenosine triphosphate-creatine phosphotransferase (EC 2.7.3.2) or creatine phosphokinase (CPK) catalyzes reversibly the transfer of phosphate between adenosine triphosphate (ATP) and creatine phosphate (Cr~P), preserving the energy rich nature of the phosphate bond, with creatine and adenosine diphosphate (ADP) serving as recipients of the high energy bond in the forward and reverse processes, reaction 1. This system is, in fact, very crucial to the conservation of the body's supply of quickly available energy produced as ATP by glycolytic or oxidative metabolism and stored in the reserve form, creatine phosphate or phosphocreatine.

$$ATP + creatine \underset{\rightleftharpoons}{\overset{CPK}{}} ADP + phosphocreatine \quad (1)$$

Ebaski, et al (11) first reported that serum creatine phosphokinase was elevated in progressive muscular dystrophy and since that time the serum or plasma activity of these enzymes has been observed to increase in various myopathies and in hereditary carriers of these diseases (8, 9, 31, 35), in hypothyroidism (19), myocardial infarction (15, 17, 25), brain, nervous tissue or cerebral vascular damage (1, 10, 24), tetanus (3, 20), cold injury (23), and acute psychoses (12, 21).

While the analysis of serum enzyme activity serves a clinically important role in each of these circumstances and thus deserves further study in its general physiological relationships, its function in the diagnosis of myocardial infarction seems best established and most frequently utilized. It is now generally recognized to respond quickly, to be more specific, and to rise to higher levels than either lactic dehydrogenase (LDH) or the transaminases after the occurrence of a myocardial infarction (2, 19, 25, 33). The analysis of CPK also has the distinct advantage of not being sensitive to hemolysis encountered during blood handling, since erythrocytes contain very low amounts of the enzyme (28); and therefore many samples that can not be analyzed accurately for transaminase or LDH activity may be utilized for CPK evaluation.

The leakage of creatine phosphokinase into serum or plasma, occurring not only under pathologically severe conditions of muscular deterioration, but under the relatively normal stress of heavy exercise (15, 32, 34), or as a result of electrical stimulations (29) in which muscle damage is absent or minimal, led to the proposal that CPK might leak into the plasma under conditions of high carbon dioxide environments. Other evidence leading to the proposal that tissue permeability might be altered under CO_2 stress included

the build-up of lactic dehydrogenase in plasma (18) and the loss of potassium from erythrocytes as a result of carbon dioxide exposure (22). A rigorous understanding of the effects of CO_2 build-up is of prime importance to the maintenance of the health of the participants in the Navy's numerous diving programs.

The results of a series of investigations evaluating the leakage of CPK into plasma and the possible effect this might have on the level of muscle creatine phosphate are reported here.

Materials and Methods

Male guinea pigs of the Hartley strain, 350-600 gm, were exposed in environmental chambers to atmospheres containing 15% carbon dioxide in the presence of 21% oxygen under one atmosphere pressure for the periods indicated in the following section. Food and water were supplied ad libidum during the exposure and blood was withdrawn into heparinized tubes from the descending dorsal aorta under nembutal anaesthesia while the animal breathed the gas mixture to which it had been exposed. After centrifugation for 10 minutes at 3000 g, the plasmas were refrigerated until analyses could be performed, usually within 1-2 hours.

Creatine phosphokinase activity was determined by a modification of the fluorometric method of Sax and Moore (26) in which a fluophor is produced in a strongly alkaline medium (4, 5). Glutathione was employed as the reducing agent at .003 M, with the concentrations of the other reagents in the reaction mixture as follows: creatine phosphate - .01 M, ADP - .003 M, magnesium acetate - .007 M and tris-acetate buffer (pH 7.1) - .15 M. For guinea pig plasma, .025 ml was incubated in a total volume of .35 ml for 30 minutes at 30°. The reaction was stopped by .5 ml of .3 N BaOH; .5 ml 5% $ZnSO_4 . 7\ H_2O$ was added and after centrifugation, .1 ml of supernate was combined with .5 ml 1% ninhydrin in ethanol and 1.5 ml 7.5% KOH. Readings were made after 5 min in an Aminco-Bowman spectrofluorometer; excitation wavelength 410 and emission wavelength 525 nm.

Creatine and phosphocreatine determinations were made on soleus muscle quickly removed and frozen in acetone-solid CO_2 on a separate series of animals not subjected to blood withdrawal. The tissue was crushed in the frozen state and promptly dropped as a powder into teflon-glass homogenizers containing preweighed amounts of cold 10% trichloroacetic acid. After weighing the tube plus sample, the powdered tissue was quickly extracted by brief homogenization and then immediately neutralized with 2 N KOH in the presence of a drop of methyl red-bromthymol blue indicator to pH 7.5-7.7. The trace of green color of the indicator mixture under the alkaline conditions of the subsequent creatine determination did not interfere with the measurement of the pink color developed by the reaction. An additional weighing provided information on the total volume of the neutralized extract. After centrifugation, a portion of the extract was hydrolyzed (13) to allow analysis of total creatine. Free creatine and creatine + phosphocreatine, or total creatine,

were determined separately in the hydrolyzed and unhydrolyzed aliquots by an automated technique described recently by simply bypassing the enzymic incubation step of the original procedure (30).

Results and Discussion

The influx of creatine phosphokinase into the plasma of guinea pigs exposed to the stressful environment as well as the approximate rate of its accumulation and decline may be obtained from Table 1. Although no increase in the plasma enzyme activity occurs after one hour of exposure, between the first and the sixth hours a significant build-up takes place. The high level, which is maintained during the first 24 hours of the exposure, has returned by the end of the third day to the upper ranges of normality in some, but not all, of the

TABLE 1 - Creatine Phosphokinase Activity in Plasma of Guinea Pigs Exposed to CO_2

Exposure		CPK Activity*	p of t**
Control	(18)#	.0997 (.0186)	---
1 hr	(9)	.0649 (.0185)	n.s.
6 hr	(11)	.3354 (.0463)	<.001
1 day	(11)	.3340 (.0717)	<.005
3 days	(18)	.7466 (.2008)	<.001
3 days "high"@	(8)	1.5471 (.2315)	<.001
3 days "low"	(10)	.1062 (.0349)	n.s.
7 days	(9)	.0675 (.0130)	n.s.

* uM / ml / min (std error of mean).
** Probability of difference from control value by Student's t test. n.s. = significant difference not proven.
\# Number of animals.
@ See text for discussion of grouping of 3-day animals into "high" and "low" categories.

guinea pigs. In order to compare the two patterns of plasma CPK response of guinea pigs at the third day of exposure, data from the entire group of animals examined at this time, as well as data from two arbitrarily selected subgroups, are shown. Since a pattern of contrasting high and low values was quite apparent at this stage, data more than twice as large as the largest control value were included in the "high" group with all other values included in the "low" group. The sharp contrast between the two groups of individual data is illustrated in Figure 1 along with comparative data from the control animals.

With the data for the 3-day animals arranged into two groups, it is very clear that in some of the animals the enzyme levels are returning to normal values while in other animals significant increases in activity are present. These data correspond to apparently related observations of Schaefer, et al (27) on blood pH and transaminase activities in which two very dissimilar patterns of response were also noted in guinea pigs exposed for three days to 15% CO_2. From the totality of such information, it seems clear that the animals that are making a successful physiological adjustment to the high CO_2 environment show diminishing signs of stress after about three days of exposure, in this case with a return toward normal plasma enzyme levels; while those animals that are unable to adjust, and will shortly succumb, show increasing stress reactions. It may be noted from the data of the 7-day-exposed animals that approximately normal enzyme levels exist in the guinea pigs surviving at this time. A loss of 50-60% of the guinea pigs surviving at three days further substantiates the distinction that should be made at or near the third day between adapting and non-adapting animals.

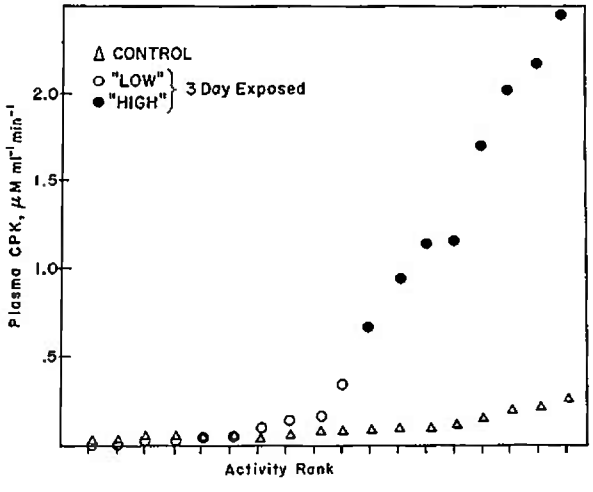

Figure 1. — Illustrating the sharp contrast between the two groups of individual data, as well as comparative data from the control animals.

Since creatine phosphokinase is involved in maintaining the energy reserves of the animals, it was of interest to investigate the reserves of creatine phosphate available in the tissues under the conditions causing such severe losses of CPK into the circulation. Table 2 shows the results of analyses of muscle concentrations of creatine and phosphocreatine in the stressed guinea pigs. Muscle tissue was analyzed as the organism's primary compartment for creatine phosphokinase as well as creatine phosphate.

TABLE 2 - Muscle Creatine and Phosphocreatine in Carbon Dioxide Stressed Guinea Pigs

Exposure		Creatine	Phosphocreatine	Ratio
Control	(15)#	4.45 (.17)*	1.77 (.29)	3.62 (.60)
1 hr	(7)	4.32 (.83)	1.93 (.45)	2.57 (.53)
4-6 hrs	(12)	4.11 (.21)	1.89 (.27)	2.69 (.40)
1 day	(11)	3.83 (.44)	2.21 (.21)	1.87 (.22)**
2 days	(10)	4.21 (.44)	2.51 (.42)	1.95 (.26)**
3 days	(10)	5.46 (.75)	2.98 (.58)**	2.53 (.52)
4-5 days	(8)	4.85 (.50)	2.40 (.61)	2.79 (.53)
7 days	(9)	4.15 (.11)	1.91 (.11)	2.20 (.11)

\# Number of animals.
* mg / g muscle (std error of mean).
** Significantly different from control, $p<.05$.

The results of these analyses indicate that there is no diminishment of creatine phosphate during the period of one to three days of exposure when the process of adaptation is occurring, apparently indicating that the synthetic ability of the enzyme in the muscle has not decreased. The loss of enzyme to the circulation does not seem of major importance to this aspect of the muscle's functioning. The data, in fact, suggest somewhat of an overcompensation in the activity of the CPK, reflected by a diminished creatine/phosphocreatine ratio or a relative increase in phosphocreatine during the critical acclimatization period. The relationship of this possible compensatory change in energy stores to the apparent increase in permeability of the tissue to CPK has not yet been explored.

Since muscle is likely to be called upon to deliver work in amounts greatly in excess of that required during periods of relative inactivity, and to carry out such work even in the presence of a relative lack of oxygen, its rich supply of CPK operates as a vital link in meeting these demands. While other tissues,

notably brain, kidney or the specialized muscle, the heart, contain significant amounts of CPK, these organs are treated as more "vital" during an emergency situation, i.e., receive a preferential supply of blood. Such organs are able to obtain energy by oxidative processes in a more normal manner even when the organism is functioning under a temporary relative shortage of oxygen with less dependence on the reserve ATP-CrP system. So different, in fact, are the requirements for CPK between the muscles and the brain that separate isozymes of the enzyme in these two tissues have been detected (6, 7, 14). The heart, a muscle, but with energy requirements more akin to those of brain, contains a mixture of the two primary isozymic forms, with at least one intermediate form composed of subunits of the two major varieties (6).

The very rich source of CPK in muscles is primarily responsible for the possibility of using this enzyme as a diagnostic tool for the detection of muscular damage in the variety of clinically significant situations which were discussed earlier. To the list of physiological and pathological conditions in which plasma CPK has been shown to be altered may now be added the stress of exposure to severely toxic levels of carbon dioxide. It may be suggested that the increased levels of CPK of plasma also arise in this situation through alterations in the permeability of the membranes of muscle tissue. It should be pointed out, however, that in another case in which high serum CPK is found, in acutely psychotic individuals, the presence of abnormal muscle fibers is related to serum CPK abnormalities (12). It may be proposed that an analysis of CPK provides a useful index to the severity of CO_2 stress with respect to an animal's overall adaptive capacity.

Work performed since the original observations reported here on enzyme leakage into serum has considerably simplified the assay procedure (30) and should help to increase its research and clinical utility. Additional studies will be required to evaluate the possible usefulness of serum creatine phosphokinase level as an indicator of human responses to a variety of other stresses.

Summary

It has been demonstrated that guinea pigs stressed by a high (15%) carbon dioxide environment at one atmosphere, containing a normal level of oxygen, respond in a one to six hour period by an influx of creatine phosphokinase into plasma. By three days of exposure, the enzyme has begun to disappear from the circulatory system except in those animals unable to satisfactorily adapt to the stress. The utilization of this analysis as a method for evaluation of an animal's adaptive characteristics or potential is discussed. The creatine phosphate supply of the muscle is not decreased by the loss into the plasma of creatine phosphokinase. A small compensatory increase in creatine phosphate stored may occur during the period of adaptation to the stress.

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13. ABSTRACT

Creatine phosphokinase, the enzyme which is currently the most sensitive tool for detecting myocardial infarction, has been shown to also be useful for the detection of serious or irreversible damage to animals exposed to stressful levels of environmental carbon dioxide. Since accumulation of carbon dioxide is a problem for which Navy divers must be constantly alert and which may affect workers in any confined environment, this analysis is suggested as a clinical test for monitoring potential damage to the health of operational personnel.

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